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IKKβ Is Required for Peripheral B Cell Survival and Proliferation[1]

Zhi-Wei Li,*† Sidne A. Omori,†‡ Tord Labuda,[2]*† Michael Karin,[3]*† and Robert C. Rickert[3]†‡

NF-κB activity in mammalian cells is regulated through the IκB kinase (IKK) complex, consisting of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ). Targeted deletion of *Ikkβ* results in early embryonic lethality, thus complicating the examination of IKKβ function in adult tissues. Here we describe the role of IKKβ in B lymphocytes made possible by generation of a mouse strain that expresses a conditional *Ikkβ* allele. We find that the loss of IKKβ results in a dramatic reduction in all peripheral B cell subsets due to associated defects in cell survival. IKKβ-deficient B cells are also impaired in mitogenic responses to LPS, anti-CD40, and anti-IgM, indicating a general defect in the ability to activate the canonical NF-κB signaling pathway. These findings are consistent with a failure to mount effective Ab responses to T cell-dependent and independent Ags. Thus, IKKβ provides a requisite role in B cell activation and maintenance and thus is a key determinant of humoral immunity. ***The Journal of Immunology,* 2003, 170: 4630–4637.**

Nuclear factor-κB designates a family of transcription factors activated by a diverse array of proinflammatory cytokines, pathogen-associated molecular patterns (PAMPs),[4] cell-bound ligands, Ags, and physical stresses (1). Expression of NF-κB target genes is essential for mounting innate immune responses to infectious microorganisms (2), but is also important for the proper development and cellular compartmentalization of secondary lymphoid organs necessary to orchestrate an adaptive immune response (3–5). In mammals, the NF-κB family consists of five members, RelA(p65), RelB, c-Rel, p50(NF-κB1), and p52(NF-κB2), that form hetero- and homodimeric sequence-specific transcriptional regulators (2). Regulation of NF-κB activity occurs at multiple levels, primarily being dependent on inducible degradation of IκB inhibitory proteins, which hold NF-κB dimers in the cytoplasm, and the proteolytic processing of the p105 and p100 precursor proteins to the mature p50 and p52 subunits, respectively (1). The processing of p105 is thought to occur constitutively (1), whereas p100 cleavage is an inducible process (6, 7). IκB degradation depends on site-specific serine phosphorylation, which triggers their polyubiquitination and proteasome-mediated destruction (1). The release of NF-κB from IκBs allows nuclear translocation and binding to specific DNA sequences (κB sites) at the regulatory regions of specific target genes (1, 2). While other mechanisms, some of which are based on post-translational modifications of Rel/NF-κB proteins, do exist, the key regulatory step in the canonical NF-κB activation pathway is phosphorylation of IκB proteins by the IκB kinase (IKK) complex (2).

Three key components of the IKK complex were identified: IKKα, IKKβ, and IKKγ (2). IKKα and IKKβ are catalytic subunits showing significant sequence similarity, whereas IKKγ (also called NEMO) is unrelated in sequence and performs a regulatory role (8). Contrary to initial predictions, however, examination of IKKα-, IKKβ-, and IKKγ-deficient mice has revealed that activation of NF-κB by most proinflammatory stimuli and PAMPs does not require IKKα, but is dependent on IKKβ and IKKγ function (9–19). $Ikk\beta^{-/-}$ mice die in midgestation due to extensive apoptosis of fetal hepatocytes culminating in liver failure (12, 14, 19). This phenotype is shared by $Rela^{-/-}$ mice and is attributed to TNF-α-induced programmed cell death, as compound mutants defective in either *Ikkβ* or *Rela* and either *Tnfr1* or *Tnfα* genes are rescued from embryonic lethality (14, 20, 21). Further examination of $Ikk\beta^{-/-}Tnfr1^{-/-}$ double-mutant mice revealed reduced thymocyte cellularity that was attributed to impaired thymocyte proliferation (22). Notably, $Rela^{-/-}Tnfr1^{-/-}$ mice can survive up to 6 mo before they succumb to opportunistic infections (20), whereas most $Ikk\beta^{-/-}Tnfr1^{-/-}$ mice die ~1 wk after birth from rampant infections (Z.-W. Li, unpublished observations). Thus, IKKβ activity is necessary for activation of innate immune responses, which depend in part on RelA (p65)-containing dimers. These findings also suggest that IKKβ is responsible for activation of several different forms of NF-κB besides those that contain RelA. Therefore, its elimination results in a larger decrease in total NF-κB activity than the mere elimination of RelA, thereby causing a more dramatic increase in sensitivity to infections. Although the general role of IKKβ and RelA in activation of innate immune response is fairly well established, there are reasons to believe that these proteins are also involved in adaptive immune responses, probably through effects on lymphocyte development and activation (23, 24).

The large decrease in total NF-κB activity caused by the loss of IKKβ provides an opportunity to learn more about the functions of NF-κB in lymphoid cells and a context for comparing previous

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[4] Abbreviations used in this paper: PAMPs, pathogen-associated molecular patterns; BrdU, 5-bromo-2′-deoxyuridine; Cre, Cre recombinase; ES, embryonic stem; F, floxed, loxP-flanked; HSA, heat-stable Ag; IκB, inhibitor of NF-κB; IKKβ, IκB kinase; LTβR, lymphotoxin β receptor; MZ, marginal zone; PC, phosphocholine; PI, propidium iodide; TD, T cell dependent; TI, T cell independent; TNFR1, TNF receptor 1; TNP, 2,4,6-trinitrophenyl; wt, wild-type.

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findings in mice deficient for specific members of the Rel/NF-κB family. Previous results have shown that the loss of RelA blocks organogenesis of Peyer's patches and lymph nodes, and splenic microarchitecture is perturbed as well (4). This effect appears to be primarily associated with stromal cell function and may involve signaling through lymphotoxin β receptor (LTβR) and TNF receptor 1 (TNFR1), as RelA/TNFR1 double-deficient hemopoietic cells can efficiently reconstitute lethally irradiated wild-type (wt) recipients (20). Targeted deletion of *Nfkb1* results in selective impairment in marginal zone (MZ) B cell formation (25), while *Nfkb2*$^{-/-}$ mice have a dramatic, but B cell extrinsic, defect in germinal center formation (3, 5). Similarly, RelB is required for homing as well as proper formation of the splenic microarchitecture, germinal center formation, and MZ organization (26). B cell differentiation in *Nfkb1*$^{-/-}$*Nfkb2*$^{-/-}$ mice is blocked at the transitional 1 (T1) stage (IgM^{high}IgD^{low}→IgM^{high}IgD^{high}) (27), similar to that in *Rela*$^{-/-}$*c-Rel*$^{-/-}$ double-mutant mice, which is blocked at the transitional 2 (T2) stage (IgM^{high}IgD^{high}) (24), whereas *Nfkb1*$^{-/-}$*c-Rel*$^{-/-}$ mice exhibit blocks in B cell activation and function (28). These findings support a role for Rel/NF-κB factors in B cell differentiation and survival, but outstanding questions of functional redundancy of NF-κB members remain. We found that lethally irradiated mice reconstituted with *Ikkβ*$^{-/-}$ fetal liver progenitor cells exhibit a complete absence of B and T cells when analyzed 6–8 wk post-transplantation (22). However, further analysis indicated that the absence of T cells is due to the increased sensitivity of IKKβ-deficient thymocytes to TNF-α-induced apoptosis, as stem cells derived from *Ikkβ*$^{-/-}$*Tnfr1*$^{-/-}$ double-mutant embryos were capable of reconstituting normal T lymphopoiesis in lethally irradiated mice (22). Nonetheless, IKKβ-deficient T cells display a proliferation defect similar to that exhibited by T cells lacking the protein kinase C θ isozyme, which is required for NF-κB activation in response to ligation of the TCR (29). As the function of NF-κB subunits is perhaps best characterized in B cells (30, 31), we sought to investigate the role of IKKβ in B cell development and function.

In general agreement with the results of deletion of individual NF-κB subunits, we found that B cell-specific depletion of IKKβ results in an increased propensity for spontaneous apoptosis in the absence of antigenic stimulation and decreased proliferative responses to a variety of B cell mitogens. Thus, IKKβ, through activation of the canonical NF-κB pathway, plays a key role in the maintenance and expansion of the B cell compartment. This function is distinct from that of the IKKα catalytic subunit, which is specifically required for late B cell maturation and formation of secondary lymphoid organs (6, 32).

Materials and Methods

Generation of Ikkβ$^{F/+}$ *and* CD19CreIkkβ$^{F/F}$ *mice*

Ikkβ$^{F/+}$ mice were generated according to the standard two-step procedure (33). A targeting vector was designed to insert *loxP* sites and the selection marker genes, *Neo*^{r} and *TK*, into the *Ikkβ* genomic locus. The floxed 1.65-kb *Hin*dIII/*Xba*I fragment of *Ikkβ* contains exon 3, which codes for the ATP binding site of the IKKβ kinase domain. A 3.4-kb *Hin*dIII fragment and a 5.2-kb *Xba*I fragment were used as 5′ and 3′ homologous arms, respectively. After electroporation, the transfected embryonic stem (ES) cells (clone GS; Incyte Genomics, St. Louis, MO) were selected with G418 (0.2 mg/ml), and the homologous recombinants were identified by Southern blot analysis of *Kpn*I-digested ES cell genomic DNA using a 1.7-kb *Xba*I/*Kpn*I fragment downstream of the 3′ homologous arm as the probe. One of seven homologous recombinants was further cultured and transfected with a Cre recombinase (Cre) expression vector to delete the selection marker genes and to generate *Ikkβ*$^{F/+}$ ES cells. Two *Ikkβ*$^{F/+}$ ES clones identified by PCR were injected into E3.5 C57BL/6 blastocysts, and the progeny with the highest degree of chimerism were crossed with C57BL/6 mice to derive lines. PCR genotyping was performed using the primers 5′-GTC ATT TCC ACA GCC CTG TGA-3′ and 5′-CCT TGT CCT ATA GAA GCA CAA C-3′, which amplify both the *Ikkβ*$^{+}$ (220-bp) and *Ikkβ*^{F} (310-bp) alleles. B cell-specific, IKKβ-deficient (*CD19CreIkkβ*$^{F/F}$) mice were generated by breeding *Ikkβ*$^{F/F}$ mice with *CD19Cre*$^{+/-}$ knockin mice expressing Cre under control of the endogenous CD19 promoter (34).

Isolation of B cells

Six- to 12-wk-old mice were sacrificed, and bone marrow, spleen, lymph nodes, peripheral blood, and peritoneal fluid were collected. Resting splenic B cells were isolated by depletion of CD43^{+} cells with magnetic beads (MACS; Miltenyi Biotec, Auburn, CA) and were used for all experiments except Southern blot analysis. For Southern blot analysis, bone marrow and spleen single-cell suspensions were purified with anti-B220 beads. Alternatively, B cells were purified by complement lysis of T cells using anti-Thy1.2 Abs (FD75 and HO11.34) and rabbit complement (Cedarlane, Hornby, Canada). Resting B cells isolated by all methods were at least 90% B220^{+} as verified by flow cytometry.

Flow cytometric analysis

Single-cell suspensions were prepared from bone marrow, spleen, lymph node, peripheral blood, and peritoneal cavity of wt (+/+) and *CD19CreIkkβ*$^{F/F}$ (F/F) mice. Bone marrow and spleen were depleted of RBC by hypotonic lysis with ACK buffer, and PBMC were isolated by Ficoll (Pharmacia Biotech, Piscataway, NJ) gradient centrifugation before staining. The Abs used for staining include anti-B220-PE, anti-B220-CyChrome, anti-Thy1.2-CyChrome, anti-CD3-FITC, anti-CD5-biotin, anti-CD23-PE, anti-CD21-FITC, anti-CD43-biotin, anti-IgM^{a}-PE, anti-IgM^{b}-PE, anti-IgD-FITC, anti-CD24/heat-stable Ag (HSA)-biotin (M1/69), and streptavidin-PerCP (all purchased from BD PharMingen, San Diego, CA) and anti-B220-allophycocyanin and streptavidin-Tricolor (Caltag Laboratories, Burlingame, CA). Flow cytometric analysis was performed using a FACScan or a FACSCalibur and CellQuest software (BD Biosciences, Mountain View, CA).

Immunohistochemistry

Cryostat sections (8–10 μm) prepared from OCT (Tissue-Tek; Sakura, Torrance, CA)-embedded spleen were dried briefly and then stored in humidified chambers overnight at 4°C. Sections were fixed in ice-cold acetone for 10 min, dried, then blocked for ≥1 h at room temperature with PBS supplemented with 5% FBS. Sections were incubated with anti-IgM^{a}-PE and anti-IgD-FITC (BD PharMingen) in blocking buffer for 2 h at room temperature, followed by three washes with PBS supplemented with 0.05% Tween 20. Slides were mounted in Gel/Mount (Biomeda, Hayward, CA) and viewed under a fluorescent microscope (E800; Nikon, Melville, NY).

Western blotting and kinase assay

Resting B cells were cultured at 2–5 × 10^{6}/ml in six-well plates for 2 h at 37°C before being stimulated with 20 μg/ml LPS (Sigma-Aldrich, St. Louis, MO) for 1 h. Total cell lysates were used for Western blotting and kinase assays as previously described (12). The Abs used are anti-IKKγ (BD PharMingen; clone 73-764) for immune precipitation; anti-IKKα (Imgenex, San Diego, CA; clone 14A231) and anti-IKKβ (Upstate Biotechnology, Lake Placid, NY; clone 10AG2) for Western blot analysis.

In vitro proliferation and apoptosis

Resting B cells (10^{6}/ml) at 10^{5}/well in triplicate in 96-well plates were cultured for 48 h in the presence of complete RPMI medium alone, IL-4 (5 ng/ml; BD PharMingen), anti-IgM (10 μg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA), LPS (20 μg/ml; Sigma-Aldrich), or anti-CD40 (5 μg/ml, clone 3/23; BD PharMingen), respectively. [^{3}H]thymidine (5 μCi/ml; Amersham Pharmacia Biotech, Arlington Heights, IL) was added for the final 16 h of the culture, and incorporation was measured by scintillation counting. For CFSE-labeled in vitro proliferation assay, B cells (10^{7}/ml) were incubated in the dark with 5 μM CFSE (Molecular Probes, Eugene, OR) for 10 min in PBS at 37°C, washed with medium, and cultured with the additives as described above for 3 days before flow cytometric analysis. To measure apoptosis, cells stimulated for 2 days under the aforementioned conditions were incubated for 10 min on ice with hypoPI buffer (0.1% sodium citrate, 0.1% Triton X-100, 50 μg/ml RNase A, and 100 μg/ml propidium iodide (PI)), and the fraction of subdiploid cells was measured by flow cytometry.

Determination of loss of the Ikkβ$^{F/F}$ *allele by real-time PCR*

Genomic DNA was isolated (DNeasy Tissue Kit, Qiagen) from resting B cells cultured for 0, 1, and 2 days. One microliter of serially diluted

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genomic DNA (12.5, 25, 50, and 100 ng) was added to 24 μl of a mixture of Sybr Green PCR Master Mix (12.5 μl), primers (1 μl at 25 μM each), and H_2O (10.5 μl). Real-time PCR was performed for 40 cycles at 95°C for 15 s and at 60°C for 1 min using an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA). Primers 5′-TAG TCC AAC TGG CAG CGA ATA C-3′ and 5′-CGC CTA GGT AAG ATG GCT GTC T-3′ were used to amplify the *Ikkβ*^{Δ} allele, primers 5′-AAG ATG GGC AAA CTG TGA TGT G-3′ and 5′-CAT ACA GGC ATC CTG CAG AAC A-3′ were used to amplify the *Ikkβ*^{F} allele, and primers 5′-ATT CGC CAA TGA CAA GAC GCT GG-3′ and 5′-GGC TGC AGT CCA CGC ACT GG-3′ were used to amplify the *Tnfr1* gene as a control. The ratio of *Ikkβ*^{Δ} and *Ikkβ*^{F} was calculated after normalization to the *Tnfr1* signal.

5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

Mice were administered BrdU (Sigma-Aldrich; 1 mg/ml plus 2% glucose) in drinking water for 3 or 6 days before sacrifice. B220^{+} B cells were isolated from bone marrow and spleen by MACS, permeabilized, and stained with anti-BrdU-FITC or an FITC-conjugated isotype-matched control Ab (BD PharMingen) in accordance with the company protocol.

In vivo lymphocyte survival assay

Splenocytes of *CD19CreIkkβ*$^{F/F}$ and *Ikkβ*$^{F/F}$ mice were labeled with CFSE (1 μM) and washed with PBS. Two hundred microliters of labeled cells at 1–2 × 10^8/ml were injected i.v. into 8 wk old 129 × C57BL/6 F_1 male mice (The Jackson Laboratory, Bar Harbor, ME). Labeled cells were also stained with anti-Thy1.2-PE and anti-B220-CyChrome and analyzed by flow cytometry before transfer. Seven days later splenocytes from the recipients were stained with anti-Thy1.2-PE and anti-B220-CyChrome and analyzed by flow cytometry. The ratio of B220^{+} to Thy1.2^{+} CFSE-positive cells was calculated and normalized to the ratio before transfer.

Immunization and ELISA

Mice were immunized with T-independent (nonencapsulated, type 2 *Streptococcus pneumoniae* (R36A), 10^8 CFU/mouse) or T-dependent (2,4,6-trinitrophenyl (TNP)-OVA, 100 μg/mouse) Ags by i.p. injection. Serial dilutions of preimmune and immune sera from immunized animals were incubated on 96-well microtiter plates coated with goat anti-mouse IgM, goat anti-mouse IgG (both from Southern Biotechnology Associates, Birmingham, AL), TNP-BSA (Biosearch Technologies, Novato, CA), or phosphocholine-BSA (gift from Dr. G. Silverman, University of California-San Diego). Plates were developed using alkaline phosphatase-conjugated anti-mouse Igκ, anti-mouse IgM, and anti-mouse IgG and the substrate *p*-nitrophenylphosphate (all from Southern Biotechnology Associates) and were read at 405 nm.

Results

Conditional deletion of Ikkβ *in B cells*

To study the role of IKKβ in B cell development, function, and survival, mice were generated bearing a conditional *Ikkβ* allele

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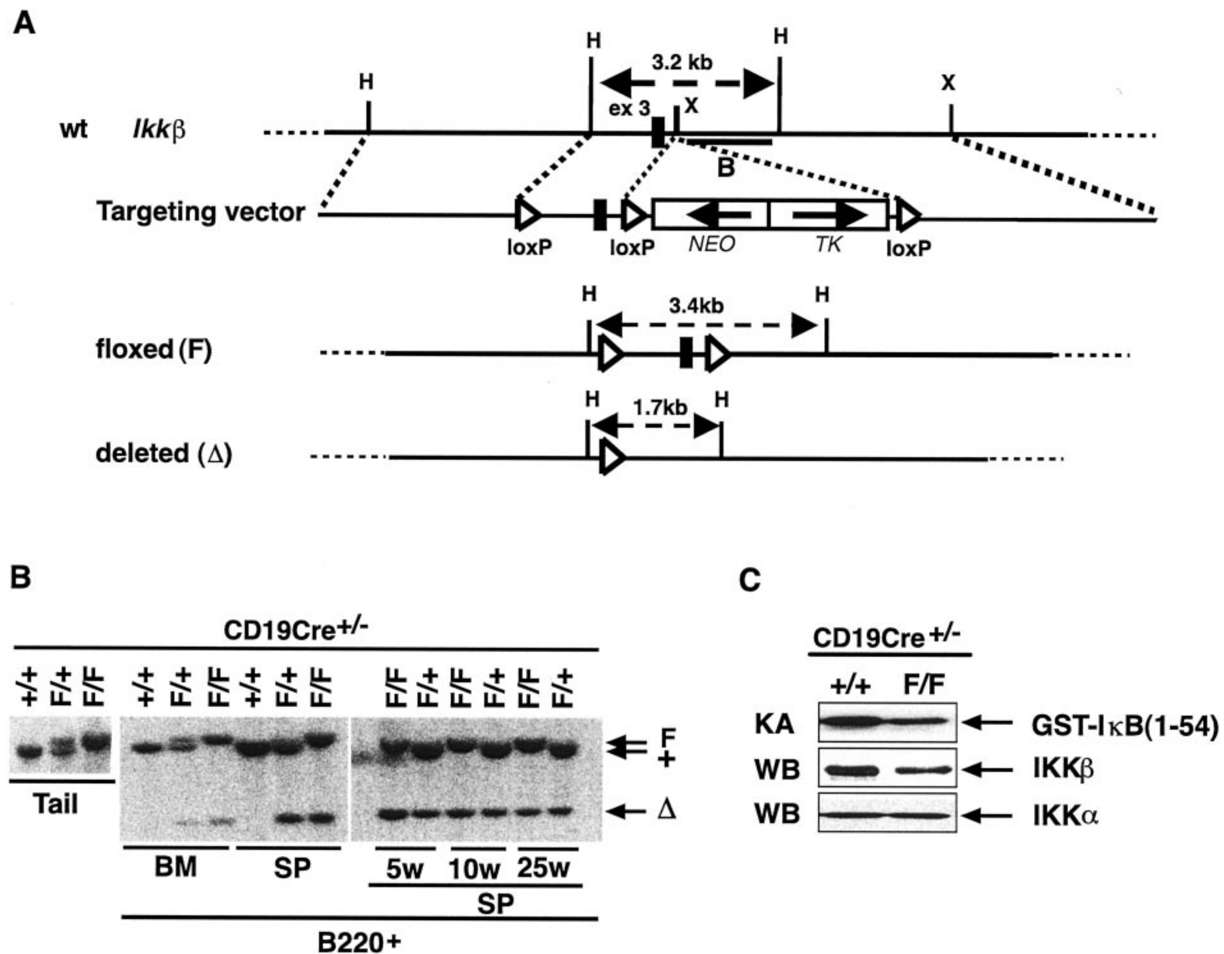

FIGURE 1. Conditional deletion of *Ikkβ* in B lymphocytes. *A*, A conditional *Ikkβ* allele (*Ikkβ*^{F}) was generated by insertion of Cre recombinase binding sites (*loxP*) into the intronic regions flanking exon 3 (ex3). Homologous recombinants were subjected to in vitro Cre-mediated deletion to remove the *NEO-TK* cassette and generate the *loxP*-flanked (floxed, F) exon 3. A nonfunctional, deleted *Ikkβ* allele (Δ) is generated in vivo in cells that express the Cre recombinase. Restriction enzymes sites are: H, *Hin*dIII; and X, *Xba*I. *B*, Genomic DNA was prepared from tail biopsies or B220/CD45R-positive B cell populations from spleen (SP) and bone marrow (BM) of CD19Cre$^{+/-}$ mice bearing the indicated *Ikkβ* allelic combinations. A Southern blot of *Hin*dIII-digested mouse genomic DNA was probed with the indicated *Xba*I-*Hin*dIII fragment (probe B) to discriminate the three distinct alleles as shown. *C*, IKK kinase activity (KA) assay and expression were examined in splenic B (CD43^{-}) cells cultured with LPS (20 μg/ml) for 1 h. The levels of IKKα and IKKβ were determined by Western blot (WB) analysis. The results are representative of three separate experiments.

($Ikk\beta^{F}$) subject to inactivation by Cre recombinase-mediated deletion (33). This was accomplished by construction of a gene targeting vector that introduces *loxP* sites into regions flanking exon 3 of the *Ikkβ* gene following homologous recombination in ES cells (Fig. 1*A*). Homologous recombinants were identified by PCR and confirmed by Southern blot analysis. Selected recombinants were subjected to transient Cre expression and selected on the basis of acquired G418 resistance. ES cells that underwent a partial deletion to generate a *loxP*-flanked (floxed, F) exon 3 were chosen for blastocyst injection and eventual germline transmission of the conditional $Ikk\beta^{F}$ allele. Mice homozygous for the $Ikk\beta^{F}$ allele were viable and phenotypically normal, confirming that insertion of *loxP* sequences did not alter *Ikkβ* gene function.

To obtain B cell-specific inactivation of *Ikkβ*, $Ikk\beta^{F/F}$ mice were bred with *CD19Cre* ($CD19^{+/-}$, Cre^{+}) mice that express *Cre* in the B lineage by virtue of targeted insertion of *Cre* into the *Cd19* locus (34). As shown in Fig. 1*B*, recombination of the $Ikk\beta^{F}$ allele is made evident by the appearance of a deletion product ($Ikk\beta^{\Delta}$) first detected in developing B cells in the bone marrow and more highly represented in the mature population of splenic B cells. B cells from homozygous ($Ikk\beta^{F/F}$) mice are rendered IKKβ-deficient in the presence of Cre, which is evident from reduced IKKβ protein levels and total IKK kinase activity in splenic B cells following LPS stimulation (Fig. 1*C*). Nonetheless, a significant proportion of B cells isolated from $CD19CreIkk\beta^{F/F}$ mice retain a functional $Ikk\beta^{F}$ allele. This finding does not appear to be due to poor deletion efficiency or specificity of Cre expression, since splenic B cells from mice heterozygous for the conditional allele ($CD19CreIkk\beta^{+/F}$) show almost complete conversion of the $Ikk\beta^{F}$ allele to the $Ikk\beta^{\Delta}$ form (Fig. 1*B*). Rather, it suggests that the loss of IKKβ poses a severe disadvantage to B cell survival, thus selecting for variants that have managed to avoid deletion. Support for this argument is provided by the assessment of deletion efficiency in splenic B cells from young, adult, and aged mice (Fig. 1*B*), showing a greater accumulation of cells bearing an intact $Ikk\beta^{F}$ allele(s) and decreased representation of the $Ikk\beta^{\Delta}$ allele in older mice.

Reduced peripheral B cell subsets in $CD19CreIkk\beta^{F/F}$ *mice*

Flow cytometric analysis of bone marrow cells indicated that B cell generation was not affected in $CD19CreIkk\beta^{F/F}$ mice (data not shown). We therefore focused on the effect of IKKβ deletion in peripheral B cells. $CD19CreIkk\beta^{F/F}$ mice were compared with $CD19CreIkk\beta^{+/+}$ cohorts to assess the composition of the B cell compartment in secondary lymphoid organs and peripheral blood. As shown in Fig. 2*A*, the relative frequency of B cells was significantly reduced in all examined peripheral compartments of $CD19CreIkk\beta^{F/F}$ mice. The most dramatic reduction (5-fold) in B cell number was seen in the lymph nodes, which primarily contain long-lived recirculating B cells (IgM^{low}, IgD^{high}, $CD23^{high}$, $CD21^{int}$). The B cell deficiency was also evident in the peritoneal B-1 cell population (Fig. 2*A*), suggesting that IKKβ is necessary for the persistence of B-1 as well as conventional (B-2) cells.

In addition to recirculating B cells present in the follicles, the splenic B cell compartment includes MZ (IgM^{high}, IgD^{low}, $CD23^{neg}$, $CD21^{high}$) B cells, which are long-lived cells that do not recirculate but occupy the region of the spleen peripheral to the marginal sinus. Formation of MZ B cells is notably impaired in $NF\text{-}\kappa B1^{-/-}$ mice and, to a lesser extent, $c\text{-}Rel^{-/-}$ and $Rela^{-/-}$ mice (24, 25). B cells from $CD19CreIkk\beta^{F/F}$ mice express lower levels of CD23, and it appears that the $B220^{high}$, $CD23^{neg}$, $CD21^{high}$ MZ B cell population is reduced (Fig. 2*B*). To substantiate this finding, we assessed the representation of MZ B cells in situ using immunohistologic staining to detect follicular (IgD^{high}) and MZ (IgM^{high}) B cells (Fig. 2*C*). In agreement with the flow cytometric analysis, B cells from $CD19CreIkk\beta^{F/F}$ mice are capable of populating the MZ, but are under-represented relative to those in control $CD19CreIkk\beta^{+/+}$ mice. Insofar as MZ B cells are

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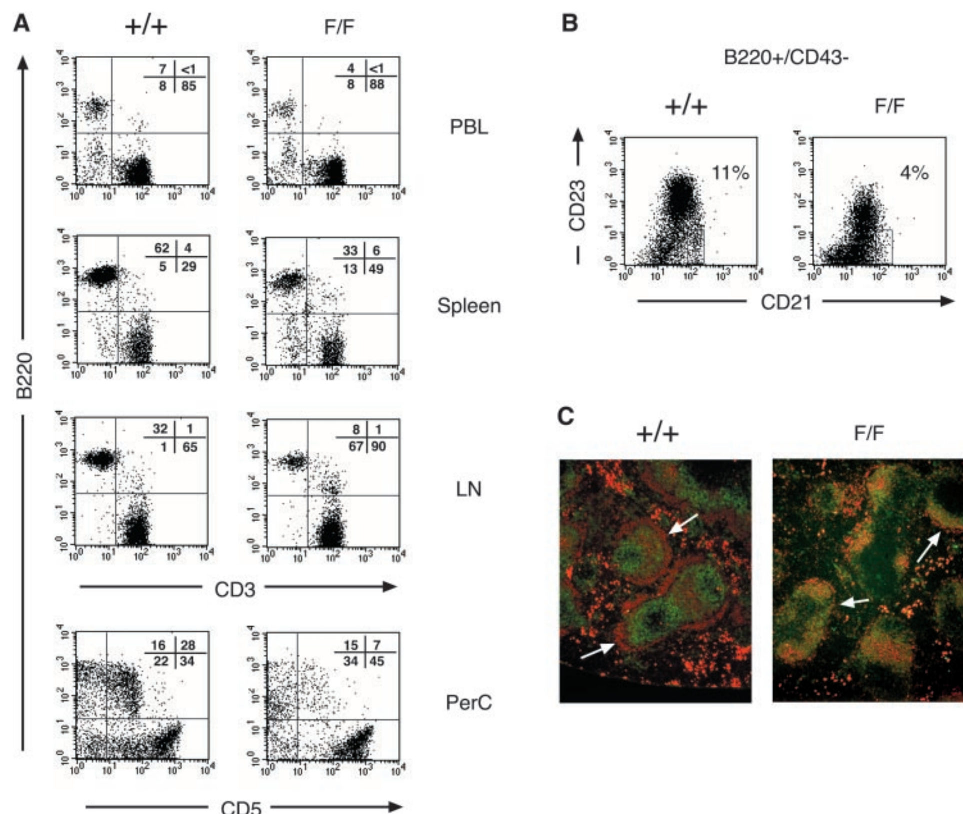

FIGURE 2. Reduced B subpopulations in the absence of IKKβ. *A*, Representative flow cytometric staining for B ($B220^{+}$) and T ($CD3^{+}$ and $CD5^{high}$) cells in peripheral blood (PBL), spleen (SP), lymph nodes (LN), and peritoneal cavity (PerC). B1a cells are $B220^{low}$ $CD5^{low}$. Conventional (B2) cells are $B220^{high}CD5^{-}$. The percentage of cells collected in the lymphocyte-gated population is indicated for each quadrant. *B*, Representative flow cytometric analysis of splenic B cells. MZ B cells (rectangle) are $CD21^{high}IgM^{high}IgD^{-}$ $CD23^{-}$. The figure shows $B220^{+}$/ $CD43^{-}CD21^{high}CD23^{-}$, not IgM^{high} IgD^{-}. *C*, Immunofluorescence analysis of spleen sections showing follicular (IgD^{high}, green) and MZ (IgM^{high}, red) B cells. The marginal zone is indicated by an arrow.

thought to be an activated subset primed by internal Ags, the observed reduction may be due to impaired activation or survival in the absence of IKKβ.

IKKβ-dependent B cell proliferation and survival in response to mitogenic stimuli

B cells undergo clonal expansion in response to microbial products and T cell-derived cytokines or upon efficient cross-linking of membrane Ig molecules. To evaluate the response of IKKβ-deficient B cells to key B cell mitogens and growth factors, purified splenic B cells from *CD19CreIkkβ*$^{F/F}$ and *CD19CreIkkβ*$^{+/+}$ mice were incubated with optimal concentrations of anti-IgM F(ab′)$_2$, LPS, anti-CD40, IL-4, or medium alone. As measured by [^{3}H]thymidine incorporation, B cells of *CD19CreIkkβ*$^{F/F}$ mice were hypoproliferative to all stimuli (Fig. 3*A*). To confirm and extend these findings, B cells were labeled with the fluorescent membrane dye CFSE and subjected to the same set of stimuli. We observed that most B cells from *CD19CreIkkβ*$^{F/F}$ mice failed to undergo cell division (Fig. 3*B*). The minor population that does respond probably represents B cells that have retained a functional *Ikkβ*$^{F/F}$ allele. Moreover, when analyzed for DNA content by PI staining, a reduced percentage of *CD19CreIkkβ*$^{F/F}$ B cells were in cycle, but an increased fraction contained subdiploid DNA indicative of apoptosis (Fig. 3*B*). IL-4 is nonmitogenic, but appears to confer increased cell survival in an IKKβ-independent manner when other costimuli are not present. These findings were confirmed by staining for annexin V as an indicator of apoptosis (data not shown). Real-time PCR analysis revealed a decreased presence of the *Ikkβ*$^{\Delta}$ allele with time in culture relative to the *Ikkβ*^{F} allele (Fig. 3*C*), suggesting that homozygous deletion of *Ikkβ* results in accelerated cell death. Thus, reduced [^{3}H]thymidine incorporation in IKKβ-deficient B cells is attributed to both reduced proliferation and increased cell death.

Loss of IKKβ results in reduced B cell survival in vivo

Most recirculating B cells in the adult animal are relatively long-lived, possessing a half-life of several weeks. In the spleen, less mature B cells representing recent emigrants from the bone marrow and mature MZ B cells are phenotypically identified as expressing high levels of IgM and HSA (CD24), whereas recirculating mature follicular B cells are IgM^{low}/HSA^{low}. In *CD19CreIkkβ*$^{F/F}$ mice, a lower proportion of splenic B cells are IgM^{low}/HSA^{low}, while the absolute number of IgM^{high}/HSA^{high} B cells is modestly decreased (Fig. 4*A* and data not shown), most likely representing the reduction in MZ B cells in *CD19CreIkkβ*$^{F/F}$ mice as described above. This finding suggests that B cells from *CD19CreIkkβ*$^{F/F}$ mice enter the peripheral B cell pool fairly efficiently, but are short-lived. To address this issue, mice were administered BrdU in the drinking water for 3 or 6 days, at which time B220^{+} cells were isolated from bone marrow and spleen and analyzed by flow cytometry for BrdU incorporation during the labeling period. The percentage of BrdU-labeled bone marrow B cells was similar in *CD19CreIkkβ*$^{F/F}$ and *CD19CreIkkβ*$^{+/+}$ mice (data not shown). However, we observed a significant increase in the percentage of BrdU-labeled spleen B cells in *CD19CreIkkβ*$^{F/F}$ mice (Fig. 4*B*), indicating that B cell turnover is increased in the absence of IKKβ. To confirm this result, total spleen cell suspensions from *CD19CreIkkβ*$^{F/F}$ and *CD19CreIkkβ*$^{+/+}$ mice were labeled with CFSE, and the relative percentages of B cells and T cells were enumerated by flow cytometry. The CFSE-labeled cells were then injected i.v. into MHC-matched, nonirradiated recipients. At 7 days post-transfer, spleens were harvested from the recipients and analyzed by flow cytometry for the relative distribution of donor (CFSE-positive) B and T cells. The B to T cell ratio was calculated and normalized to that measured before transfer. As indicated by the unaltered B/T cell ratio in transferred and recovered *CD19CreIkkβ*$^{+/+}$ cells, wild-type T and B cells showed a similar degree of survival over the 7-day period. However, a precipitous and specific loss occurred with transferred *CD19CreIkkβ*$^{F/F}$ B cells (Fig. 4*C*), thus supporting the idea that IKKβ-deficient B cells are prone to apoptosis, resulting in their rapid disappearance.

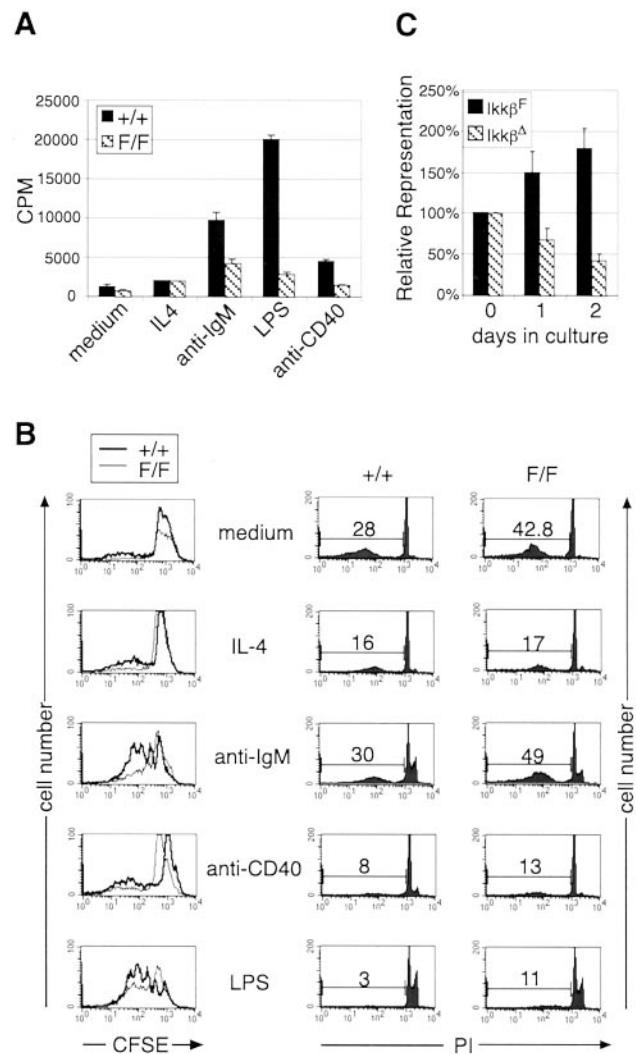

FIGURE 3. IKKβ-dependent B cell proliferation and survival in response to mitogenic stimuli. *A*, Purified splenic B cells were cultured for 2 days in the presence of medium alone, IL-4 (5 ng/ml), anti-IgM F(ab′)$_2$ (10 μg/ml), LPS (20 μg/ml), or anti-CD40 (5 μg/ml). [^{3}H]thymidine was added for the final 16 h of culture. Results shown are representative of three experiments performed in triplicate. *B*, Splenic B cells were preincubated with 5 μM CFSE and subjected to the same stimuli as above. Cells were permeabilized at the end of the treatment period and stained with PI to enumerate subdiploid, apoptotic cells (percentages indicated). *C*, Loss of the *Ikkβ*$^{\Delta}$ allele in cultured B cells. Real-time PCR was performed with primers to amplify the *Ikkβ*^{F} allele and its deletion product (*Ikkβ*$^{\Delta}$) using DNA isolated from cultured B cells. Data shown are the relative representation of the *Ikkβ*$^{\Delta}$ and *Ikkβ*^{F} alleles at each time point after normalization to the signal derived from the *Tnfr1* gene. Results shown are the averages of four separate experiments.

IKKβ is necessary for T cell-independent (TI) and T cell-dependent (TD) Ab responses

Measurement of serum Ig levels by ELISA revealed decreases in the basal levels of IgM and IgG in *CD19CreIkkβ*$^{F/F}$ mice (Fig. 5*A*). The reduced IgM levels are probably indicative of the reduced

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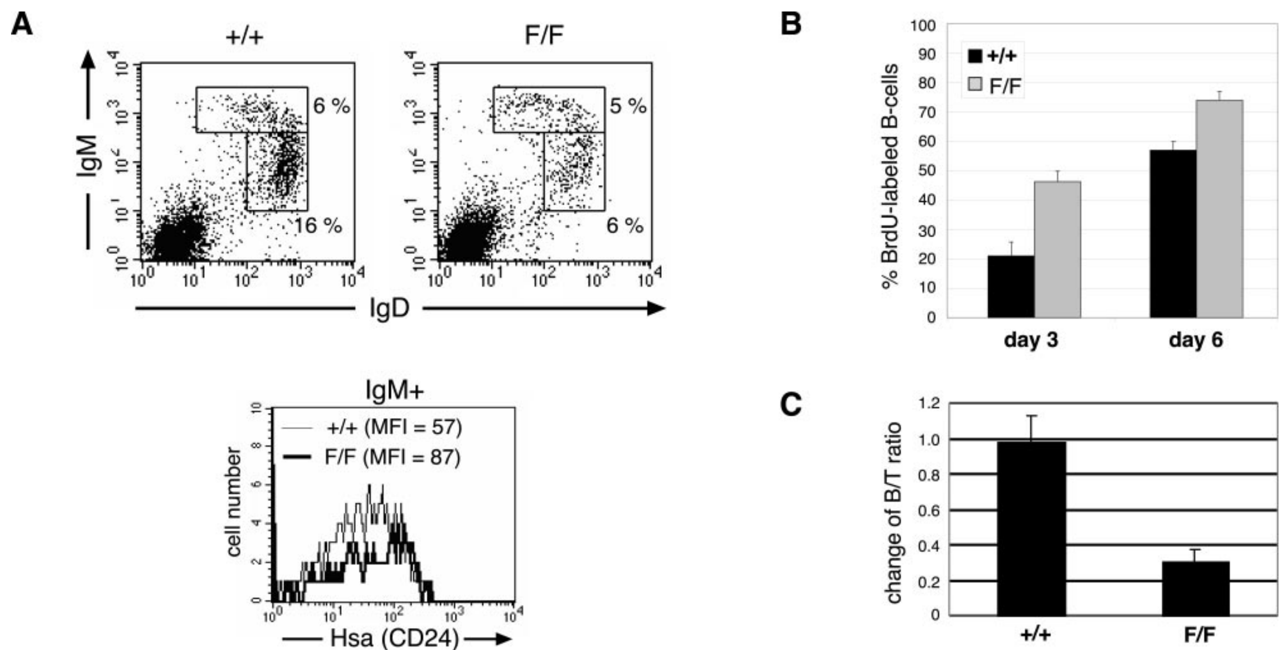

FIGURE 4. Loss of IKKβ results in reduced B cell survival. *A*, Decreased percentages of mature recirculating B cells in *CD19CreIkk*$\beta^{F/F}$ mice. Dot plots show the relative frequencies of IgM and IgD expression in *CD19Cre* mice that is either *Ikk*$\beta^{+/+}$ or *Ikk*$\beta^{F/F}$. Histograms show mean fluorescence intensity (MFI) of HSA expression on IgM-positive cells. *B*, Increased turnover of *CD19CreIkk*$\beta^{F/F}$ B cells as evidenced by increased frequencies of BrdU-positive cells following short term continuous BrdU treatment. B cells that have incorporated BrdU were identified following permeabilization and staining with an anti-BrdU-FITC Ab. *C*, Reduced survival of transferred CFSE-labeled *CD19CreIkk*$\beta^{F/F}$ B cells relative to *CD19CreIkk*$\beta^{+/+}$ B cells. B cell survival in vivo was determined by the relative ratio of B cells and T cells recovered at 7 days post-transfer. Results shown are the averages from four mice transferred with splenocytes of *CD19Cre*$^{+/-}$ mice that are either *Ikk*$\beta^{+/+}$ or *Ikk*$\beta^{F/F}$.

B-1 and MZ B cell populations that contribute a significant proportion of the natural Ab titer. To examine the responsiveness of *Ikk*$\beta^{F/F}$ mice to intact bacteria, mice were immunized with nonencapsulated, type 2 *S. pneumoniae* (R36A) and monitored for phosphocholine-specific Ab production 7 days postimmunization. We found that *CD19CreIkk*$\beta^{F/F}$ mice mounted a poor response to R36A (Fig. 5*B*), consistent with the reduction in MZ and B-1 cells and minimal responses to anti-IgM stimulation in vitro. The Ab response to TD Ags was also examined by immunization with TNP-OVA. We found that *CD19CreIkk*$\beta^{F/F}$ mice were greatly impaired in the production of TNP-specific Abs (Fig. 5*C*), which is consistent with an impaired response to anti-CD40 and a general B cell survival defect. Of note, the reduction in TD Ab production was more dramatic than predicted from the fraction of resting splenic B cells that retained IKKβ expression. Thus, in agreement with the in vitro stimulation data, it appears that in addition to decreased survival of IKKβ-deficient B cells, their decreased mitogenic response contributes to the overall decrease in Ab production.

Discussion

Characterization of the IKK complex led to the identification of two catalytic subunits, IKKα and IKKβ, which in vitro can phosphorylate IκB proteins at sites that are required for ubiquitin-dependent IκB degradation and translocation of NF-κB dimers into the nucleus (1). Surprisingly, however, targeted deletion of the *Ikk*α gene revealed minimal defects in NF-κB activity induced by proinflammatory stimuli, but an absolute requirement for IKKα in epidermal differentiation (11, 13, 18). By contrast, *Ikk*$\beta^{-/-}$ mice die in midgestation due to TNF-α-induced apoptosis of hepatocytes (12, 14, 19). This phenotype is shared by *Rela*$^{-/-}$ mice (36) and underscores the important antiapoptotic function of NF-κB (37). When the *Ikk*β *null* mutation is bred onto the *Tnfr1*$^{-/-}$ background, double-mutant mice are rescued from embryonic lethality (14, 22). However, most of these mice die before weaning as a result of severe infections (Z.-W. Li, unpublished observations), thus precluding the analysis of *Ikk*β function in the adult. We overcame this difficulty through the generation of a conditional *Ikk*β allele and used this system to probe *Ikk*β function in B lymphocytes. We found that *Ikk*β is required for B cell survival and mitogenic responsiveness to diverse stimuli, including those that act via the B cell receptor.

To ablate IKKβ function in B cells, mice expressing a floxed *Ikk*β^{F} allele were generated and bred with mice expressing Cre recombinase under transcriptional control of the CD19 promoter (34). In this system the onset of *Cre* expression occurs at the pro-B cell stage and continues throughout B cell development and differentiation. Correspondingly, *CD19CreIkk*$\beta^{F/F}$ mice show a strong reduction in peripheral B cell numbers that affects all B cell subsets, including MZ and B-1 cells. By contrast, early B cell development in the bone marrow was not affected, as determined by flow cytometry and BrdU labeling experiments. These findings are consistent with the phenotype of *Nfkb1*$^{-/-}$*Nfkb2*$^{-/-}$ and *Rela*$^{-/-}$*c-Rel*$^{-/-}$ double mutants, which show intact generation of IgM-positive immature B cells, but a strong impairment in the generation or maintenance of mature B cells (24, 27). Thus, while early B cell development in bone marrow is intact, B cell homeostasis in peripheral lymphoid tissues of *CD19CreIkk*$\beta^{F/F}$ mice is impaired, resulting in increased turnover of IKKβ-deficient B cells.

A significant proportion of the mature B cells in *CD19CreIkk*$\beta^{F/F}$ mice were found to retain at least one functional *Ikk*β allele. Since B cells from *CD19CreIkk*$\beta^{+/F}$ mice showed complete deletion of the floxed allele, it became evident that Cre-mediated deletion of the *Ikk*β^{F} allele is efficient and that strong selection is imposed to deplete *CD19CreIkk*$\beta^{F/F}$ B cells that had deleted both *Ikk*β alleles.

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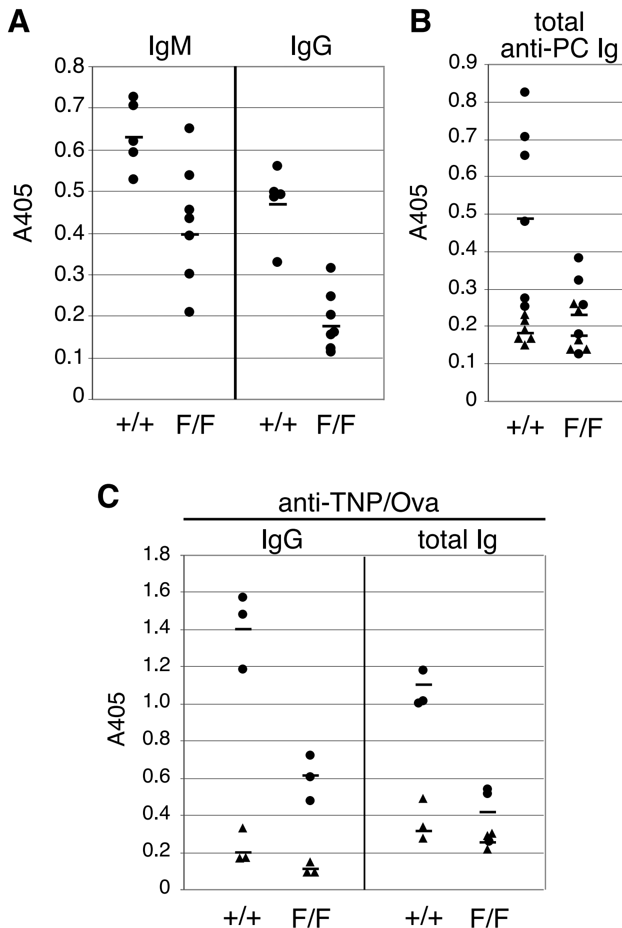

FIGURE 5. IKKβ is necessary for TI and TD Ab responses. *A*, Preimmune IgM and total IgG levels as determined by serum ELISA. Each data point represents an individual mouse (n = 5–7), and mean values are indicated by a bar. *B*, Serum titers of anti-PC specific Igκ Ab 7 days after immunization with *S. pneumoniae* (R36A). ▲, Preimmune sera; ●, immune sera. Each data point represents an individual mouse (n = 5–6), and mean values are indicated by a bar. *C*, Serum titers of anti-TNP specific Igκ and IgG Abs 7 days after immunization with TNP-OVA. ▲, Preimmune sera; ●, immune sera. Each data point represents an individual mouse (n = 3), and mean values are indicated by a bar.

The interpretation that IKKβ is essential for B cell survival is supported by our finding that B cells from *CD19CreIkkβ^F/F^* mice showed increased turnover in vivo, as measured by BrdU labeling kinetics and tracking of transferred CFSE-labeled cells. Moreover, cultured *CD19CreIkkβ^F/F^* B cells showed a considerable increase in the rate of apoptosis, as measured by DNA staining and as inferred by the decreased presence of B cells bearing an $Ikk\beta^{\Delta}$ allele. These in vitro studies indicate that IKKβ-deficient B cells possess an intrinsic defect in survival as opposed to increased susceptibility to an extrinsic factor. In the specific case of TNF-α-induced apoptosis, this conclusion is supported by our findings that IKKβ-deficient B cells from *CD19CreIkkβ^F/F^* mice bred onto the $Tnfr1^{-/-}$ background do not show any increased survival capacity relative to B cells from *CD19CreIkkβ^F/F^* mice (Z.-W. Li, unpublished observations). Thus, IKKβ-dependent protection from TNF-α may be necessary for thymocyte survival, but does not appear to be applicable to B cells. These findings suggest that the basal level of nuclear NF-κB in resting B cells is biologically significant and is required for continued synthesis of antiapoptotic proteins (37).

NF-κB is activated downstream of a diverse group of surface proteins that include members of the Toll, TNF, and Ig receptor families (38, 39). We found that IKKβ is essential for signaling by representative members of these families, in that $CD19CreIkk\beta^{F/F}$ B cells showed severe defects in the proliferative response to anti-IgM, LPS, or anti-CD40 stimulation. These stimuli activate NF-κB by distinct upstream adaptors and kinases, not all of which are fully elucidated (38, 39). However, all these stimuli appear to promote B cell proliferation by using the IKKβ-dependent canonical NF-κB activation pathway that cannot be fully activated by IKKα (2). Reduced $CD19CreIkk\beta^{F/F}$ B cell proliferation as measured by [^{3}H]thymidine incorporation does not appear to be due simply to increased cell death. We derive this conclusion from the observation that gating on live CFSE-labeled $CD19CreIkk\beta^{F/F}$ B cells reveals little evidence of cell division, and the finding that stimulated, viable $CD19CreIkk\beta^{F/F}$ B cells do not show increased cell size. These findings are in agreement with in vivo defects in Ab responses to both TI-2 and TD Ags. Together, these data indicate that IKKβ-dependent activation of NF-κB is necessary for proliferation induced by polyclonal mitogenic stimuli and for Ag-driven clonal expansion.

The observed defects in $CD19CreIkk\beta^{F/F}$ mice are most consistent with the combined loss of *Nfkb1* and *c-Rel* as well as *Rela* and *c-Rel*. These mice also have reduced follicular, B-1, and MZ B cell populations; increased B cell turnover; and a failure to proliferate in response to anti-IgM or LPS (24, 28). Single mutants in *c-Rel* or *Nfkb1* show less severe and selective defects (40, 41), and $c\text{-}Rel^{-/-}$ B cells are not prone to apoptosis in the quiescent state. However, mitogenic stimulation with anti-IgM, LPS, or anti-CD40 results in increased apoptosis due to an apparent failure to upregulate Bcl-x_L and A1 (42–44). $Nfkb1^{-/-}$ B cells are also impaired in responding to mitogenic stimuli, but have an additional defect in cell survival in the quiescent state. By contrast, RelA is not necessary for B cell autonomous functions in vivo (4), and $Rela^{-/-}$ B cells respond normally to LPS, CD40, and B cell receptor stimulation in vitro (23). Instead, RelA appears to be required in nonhemopoietic tissues for the formation and organization of secondary lymphoid tissues (4). In the absence of both *Nfkb1* and *Rela,* early B cell development is halted; however, this defect is not B cell autonomous (45).

This report and other recent findings from mice deficient for IKKα or expressing mutated forms of IKKα or IKKβ complex (6, 32, 46, 47) have led to a new appreciation for the individual functions of these components in B cells. Loss of *Ikkα* also results in reduced cellularity of the B cell compartment in fetal liver-reconstituted mice (6, 32). However, the nature and severity of the defect are more closely allied with a partial arrest in B cell maturation rather than acute responses to inflammatory stimuli (6, 32). Indeed, IKKα is not required for activation of the canonical NF-κB signaling pathway in B cells and instead is required for the NIK-dependent induction of p100 processing (6). Thus, it is possible that hyporesponsiveness of $Ikk\alpha^{-/-}$ B cells to mitogens is attributed to their immature nature. Unlike IKKβ-deficient mice, $Ikk\alpha^{-/-}$ chimeric mice exhibit striking defects in lymph node organogenesis and splenic cellular architecture (6, 32). These defects may be B cell dependent, yet rely mainly upon the maintenance of a proper stromal environment. A major challenge for the future entails the identification of the target genes responsible for the distinct biological functions of the two NF-κB activation pathways.

While this paper was under review, Pasparakis et al. (48) reported on the role of IKKβ in B cells using a similar genetic approach. In agreement with our findings, these authors show that total peripheral B cell numbers are reduced in the absence of IKKβ

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due to specific losses in the follicular and MZ B cell compartments. Using in vivo anti-IL-7R mAb treatment, they show that the remaining peripheral B cells have either only recently deleted the second $Ikk\beta^F$ allele and still presumably retain some IKKβ protein or have escaped deletion and still retain one intact $Ikk\beta^F$ allele. Importantly, the data presented by Pasparakis et al. (48) and our present study clearly demonstrate that in addition to its established role in suppression of receptor-induced apoptosis (37), the IKKβ-dependent canonical NF-κB signaling pathway is also of importance for sustaining nonstimulated resting B cells. Additionally, we have shown that IKKβ may also play a role in B cell proliferation and activation in response to various mitogenic stimuli and both TI and TD Ags. Dissecting IKKβ-dependent survival vs activation mechanisms remains a challenging topic for future study.

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